Early events involved in barley response to phytopathogenic fungi with different lifestyles

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Abstract

Broad spectrum of different fungal pathogenic lifestyles can infect barley plants, most of which are responsible for significant annual crop losses. Understanding of gene expression that take place at the earliest stages of infection would be a necessary step for describing the initial mechanism between barley and the pathogen interactions. The purpose of the present work was to monitor the expression of some well-identified genes *PR1, PR2, PR3,PR5*, *PAL* and *SGT1* during interaction of resistant barley plants with three economically important diseases *viz*. spot blotch (*Cochliobolus sativus*), scald (*Rhynchosporium secalis*) and powdery mildew (*Blumeria graminis*) at very early stages of disease development. Data demonstrated a remarkable contradiction in the gene expression patterns between barley and pathogens interactions 12 and 24 hours post inoculation (hpi), and all of them showed significant differential expressions compared to the control plants. The most significant differences were balanced in *SGT1* expression which was 3.86 (*C. sativus*) and 2.5 (*R. secalis* and *B. graminis*) folds higher at 12 hpi as compared with the corresponding control treaments. The results revealed that barley plants activated various resistance mechanisms against the three pathogens 12 hpi and increased dramatically at 24 hpi, and the same defense-related genes expression were changed in adaptation to the each fungus. Overall, this work provides insight into a signaling pathway that accounts for classical gene expression changes at very early times of infection, elicited during barley interaction with fungal pathogens having various lifestyles.

Keywords: Barley, Defense response, Powdery mildew, Quantitative PCR, Scald, Spot blotch.

Introduction

Research into plant defense responses at early infection stages by fungal pathogen is considered very important for pathogenesis studies and plant breeding programs. Recently, with the applying proteomics, genomics and transcriptomics techniques, different biochemical events can occur at very early stages of plant pathogen interactions (Kumar and Kirti, 2015; Zhao *et al.,* 2019). Since plants have innate immune systems that can at early stages distinguish the presence of pathogens and thus activate defense responses (Chowdhury *et al.,* 2017). Therefore, the biochemical mechanisms involved in plant resistance to these fungal pathogens are highly dynamic inducing both direct and indirect defense responses. As a consequent, increasing our understanding of these protective mechanisms is still needed.

It has been reported that plant leaves motivate the defense mechanisms 3 hpi in maize inoculated with *Colletotrichum graminicola*, 24 hpi in wheat challenged with yellow rust, and 12 hpi in rice (Liu *et al.,* 2016). These examples showed that plants responses against pathogens started rapidly after coming in touch with each other and then various signal pathways to achieve disease resistance were triggered. Therefore, defense mechanisms and signaling pathways remain to be investigated.

Spot blotch caused by the necrotrophic fungus *Cochliobolus sativus*, scald caused by the *hemibiotroph* fungus *Rhynchosporium secalis,* and powdery mildew caused by the biotrophic fungus *Blumeria graminis* f. sp. *hordei*, are among the most important fungal pathogens of barley causing economic crop losses globally (Gangwar *et al.,* 2018). Barley *plants* have developed a complex defense system *against these diverse pathogens*; however, the molecular events at very early stages of infection are not yet completely understood (Glazebrook, 2005).

Changes in the expression levels of a large number of defense-related genes can be estimated in barley plants during early stages of fungal pathogen infection, which considered a key defense genes (Stephens *et al.,* 2008; Shen *et al.,* 2017). Therefore, it is useful to improve our understanding concerning the changes of these genes in barley challenged by the four fungal pathogens with different *lifestyles.* Quantitative PCR (qPCR) is reported to be an effective method for measuring the relative expression level of particular genes in plant species infected with different fungal pathogens (Nolan *et al.,* 2006; Derveaux *et al.,* 2010).

Activation defense responses have been considered to be as one of the first reaction levels that usually noticed after disease infection (Kumar *et*

al., 2002). A number of plant defense-related genes including *PR* genes like *PR1, PR2, PR3, PR5*, *PAL* and *SGT1* were identified after fungal pathogen infection. These genes having various functions for instance systemic acquired resistance (*PR1*), beta-1,3-glucanase (*PR2*), chitinases (*PR3*), thaumatin like (*PR5*), secondary phenylpropanoid metabolism (*PAL*) and R *protein* accumulation (*SGT1*). However, since *PR1, PR2, PR3* and *PR5* genes coding key enzymes in the *PAL* and due to these functional roles they were chosen in this work.

There is still a great deal to be learned concerning the defense mechanisms at very early stages of barley infection with different fungal pathogenic lifestyles (Al-daoude *et al.,* 2016; Jawhar *et al.,* 2017a, b). Therefore, we evaluated here the expression changes of some important genes *PR1, PR2, PR3, PR5*, *PAL* and *SGT1* at very early interaction periods 12 and 24 h after pathogens begin to contact with barley plant leaves surfaces.

Materials and Methods

Experimental design

The barley cv. Banteng from Germany was considered to be highly resistant to all *B. graminis* and *C. sativus* isolates available so far under extensive field trials for 15 years under greenhouse and field conditions (Arabi and Jawhar, 2004; 2012), therefore, it was used in the present work. Seeds were grown in plastic pots filled with peat moss, 10 seeds/pot with three replicates of each treatment. They were kept in a growth chamber at temperatures 18–22 ºC and a relative humidity of 90%.

Inoculation with *C. sativus*

The high virulent isolate of *C. sativus* (pt4) described by Arabi and Jawhar (2004) was used in this study. Culture was grown on Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) at 20 °C for 10 days in an incubator. Seedlings were uniformly inoculated with a suspension 2×10^4 conidia mL^{-1} as reported previously by Arabi and Jawhar (2004).

Inoculation with *B. graminis*

Barley seedlings were infected with the virulent *B. graminis* conidiospores isolate (Pt1m) as described previously by Arabi and Jawhar (2012). Plants were placed under growth chamber, while control uninoculated plants were transferred to another growth chamber to keep away from infection with *B. graminis*.

Inoculation with *R. secalis*

The most virulent Syrian pathotype *R. secalis* (Rs46) described by Arabi *et al.* (2010) was used in this study. Mycelia was grown on Petri dishes containing lima bean agar (LBA), and the fungal suspension used to inoculate barley seedling was adjusted to 0.5×10^6 (Zadoks *et al.,* 1974).

RNA extraction and cDNA synthesis

Third barley leaves were collected at 12 and 24 hpi using liquid nitrogen, and control non-inoculated plants were collected at each time period. mRNA from each sample was isolated using Nucleo Trap mRNA mini kit (Macherey-Nagel, Düren, Germany). cDNA was synthesized according to Quanti Tect Reverse Transcription Kit (Qiagen, Germany).

Quantitative real-time PCR (qPCR)

Expressions of the selected genes *PR1, PR2, PR3, PR5*, *PAL* and *SGT1* were performed in Step One Plus using SYBR Green Master Mix (Roche, USA). The PCR primer sequences are shown presented in Table 1. The threshold cycle (Ct) was determined according to Livak and Schmittgen (2001). Raw data of fluorescence levels were checked by qPCR dissociation curve analysis using StepOne™ Software v2.3. Tukey's test was used for statistical significance at 0.05 level.

Results and Discussion

In order to investigate the defense mechanisms present in resistant barley plants at very early stages of infection with three fungal pathogens having different lifestyles *C. sativus, R. secalis* and *B. graminis* (Table 2), the expression of six genes *viz. PR1, PR2, PR3,PR5*, *PAL* and *SGT1*was assayed in the resistant barley cv. Banteng. Oligonucleotides designed from NCBI database demonstrated *consistent* results across replicates and gave differential amplification profiles (Table 1).

Results revealed significant changes in the gene expressions after infection with the three pathogens 12 and 24 hpi in barley resistant plants as compared with mock inoculated controls. However, the expression of the same genes was changed due to each fungus (Fig. 1). The most noticed differences were recorded in *SGT1* gene expression, which was highly regulated for all the pathogens which were 3.86 (*C. sativus*) and 2.5 (*R. secalis* and *B. graminis*) folds higher 12 hpi as compared with the respective controls. These results indicate that this gene has different roles in response to various biotic pressures. Similarly, *SGT1* was bound to enhance the resistance of *Nicotiana benthamiana* to the necrotrophic fungus *Botrytis cinerea* (El Oirdi and Bouarab, 2007), while compromised resistance was observed when barley and *H. villosa* infected with the biothoph *B. graminis* fungus (Shen *et al.,* 2003; Xing *et al.,* 2013).

Interestingly data showed that some of the studied genes were related with a multi-gene resistance which removes the current credence that identical responses are implicated in defense mechanisms to different fungal pathogenic lifestyles. For instance, *PR1, PR2* and *PR3* expressions were higher for both the necroptophic *Cs* and hemibiotrophic *Rs* as compared with biotrophic ones

B. graminis (Fig. 1), and probably is speedily targeting secondary mycelia growth of *C. sativus* and *R. secalis* than *B. graminis* This variation might be attributed to the fact that biotrophy demands a suitable period to suppress programmed cell death over effector secretion. Spanu and Panstruga (2017) reported that the high stress of plant defenses might motivate the alteration from biotrophy to necrotrophy. On opposite, the change to necrotrophy and hemibiotrophic such as *C. sativus* and *R. secalis* could also be attributed to the fungal requires for improved nutrient acquirement (Kabbage *et al.,* 2015). It has also reported that biotrophic *Uromyces vignae* and hemibiotrophic *Mycosphaerella graminicola* have suppressed the host defenses post fungal pathogen infection through the biotrophic phase (Doehlemann *et al.,* 2008). The *PRs* functions in plant cell walls have been well documented (Golshani *et al.,* 2015).

On the other hand, *PAL* expression started also 12 hpi and slightly increased after 24 hpi (Fig. 1). However, Huang *et al.* (2010) reported that domination of phenylalanine to transcinnamate is a crucial regulation point between primary and secondary metabolism. This fact might be the reason of barley cell wall leakage during infection by pathogen. Similarly, Kervinen *et al.* (1998) found an early increase in *PAL* expression in response of barley to fungal pathogens and elicitor treatments.

Our results can be supported by the recent works using the development of proteomics, genomics and transcriptomics methods that proved noticeable changes in wheat gene expressions at very early stages of *Fusarium graminearum* infection (Goswami *et al.,* 2006), and comparative transcriptomics analysis rice and *Magnaporthe oryzae* (Li *et al.,* 2015).

Conclusion

Collectively, this work suggested that in barley resistant plants various defense mechanisms can be activated to strengthen its necrotrophic *C. sativus*, hemibiotrophs *R. secalis* and biotrophic *B. graminis* resistance at very early stages of infections 12 and 24 hpi, and that the same defense-related genes expression were changed in adaptation to the each pathogen. The most observed variations were detected in *SGT1* expression which was higher at 12 hpi as compared with controls. The data could be in line with the well-accepted notion that defense strategies are very intense in barley resistant plants.

Table 1: Properties and nucleotide sequences of primers used in this study.

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
EF1α	Elongation factor-1 Alpha	AT1G07920	TGGATTTGAGGGTGACAACA CCGTTCCAATACCACCAATC	167
PR1	Pathogen-related protein	AY005474	ACTACCTTTCACCCCACAACGC TTTCTGTCCAACAACATTCCCG	182
PR2	Beta1,3-glucanase2	AT3G57260	TCATCCCTGAACCTTCCTTG GGGGCTACTGTTTCAAGCAA	193
PR3	Basic Chitinase	AT3G12500	GGGGCTACTGTTTCAAGCAA GCAACAAGGTCAGGGTTGTT	187
PR5	Pathogen-related protein S	AT1G75040	GGAGACTGTGGCGGTCTAAG GCGTTGAGGTCAGAGACACA	197
PAL	Phenyl alanine amino lyase	AT2G14610	CCATTGATGAAGCCAAAGCAAG ATGAGTGGGTTATCGTTGACGG	123
SGT1	–	AF439974	GGCTGTTGCTCCTGCTACATCTTC CGAGGCTGGAAATGGTATGGTTC	161

Table 2: Early interaction between barley and three fungal pathogens used in the study.

Pathogen	Hours after inoculation		Reference
	12	24	
C. sativus	Germination spores with only a small percentage forming hyphal appressoria.	Appressoria contact with anticlinal epidermal cell walls and stomata.	Rodríguez-Decuadro *et al.* (2014)
R. secalis	Germination spores and produce germ tubes, at from which appressoria form in response to stimuli from the leaf surface.	From the appressoria, fungi directly penetrates the cuticle above epidermal cells by means of penetration pegs rather than entering the leaf through stomata	Jones and Ayres (1974)
B. graminis	Grows on the epidermis and infecting cells by appressoria	Haustoria aformation onwards within host cells, enabling the fungus to feed	Zhang *et al.* (2005)

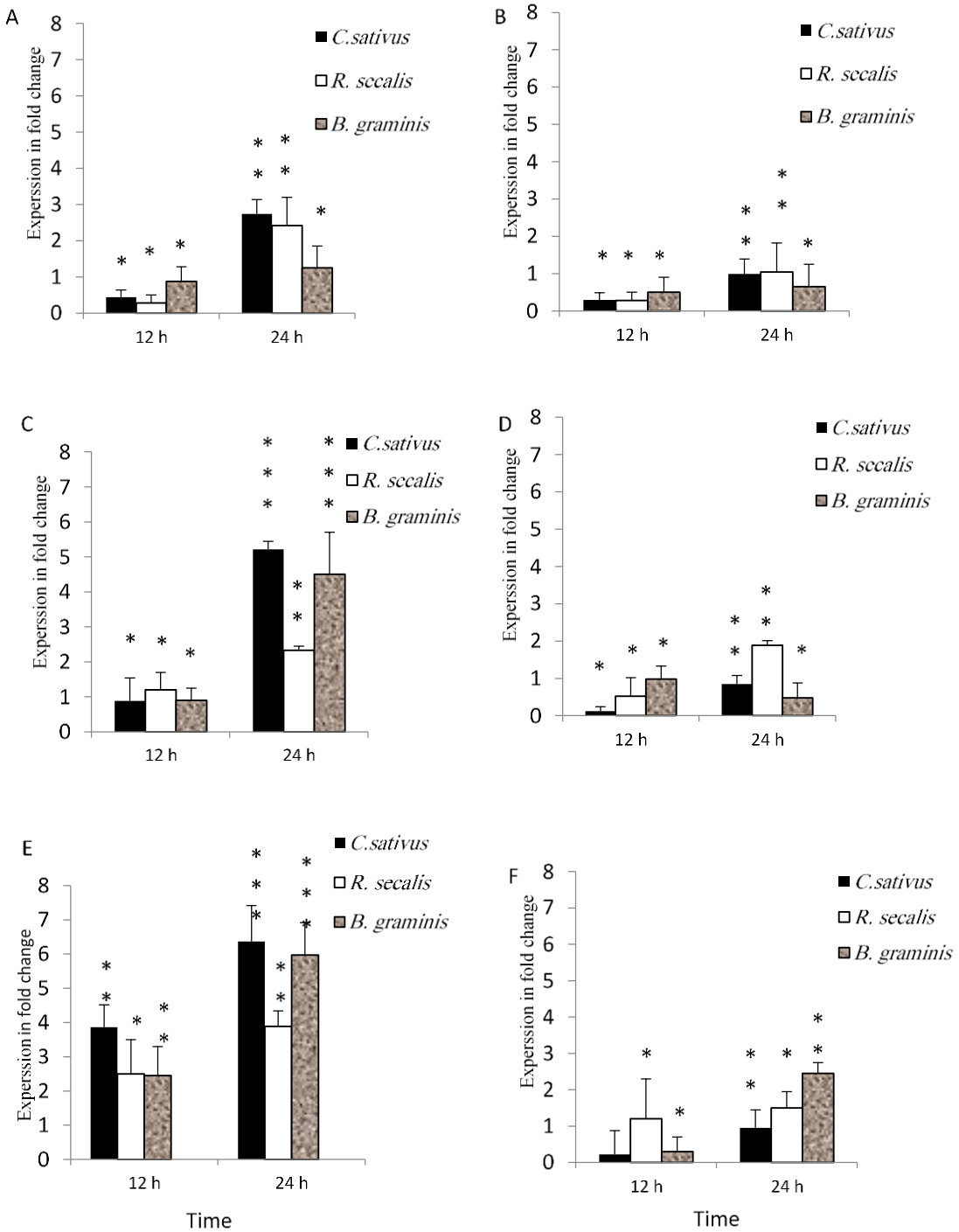

Fig. 1: Relative expression profiles of six genes; *PR1* (A), *PR2* (B), *PR3* (C), *PR5* (D), *PAL* (E) and *SGT1*(F) in the resistant barley cv. Banteng during 12 and 24 h following infections with three pathogens (*C. sativus*, *R. secalis* and *B. graminis*). Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 0). Significance at *$P<0.05$, **$P<0.01$ and ***$P<0.001$ within each genotype during different periods comparing with the control.

References

Al-Daoude A, Shoaib A, Al-Shehadah E, Jawhar M, Altahan AA, 2016. ArabiMIE. Barley transcript regulation as *Rhynchosporium seclais* changes its trophic lifestyle. *J. Plant Pathol*., **97**: 1-6.

Arabi MIE, Jawhar M, 2004. Identification of *Cochliobolus sativus* (spot blotch) isolates expressing differential virulence on barley genotypes in Syria. *J. Phytopathol.,* **152**: 461-464.

Arabi MIE, Jawhar M, 2012. Expression of resistance to *Blumeria graminis* in barley genotypes (*Hordeum vulgare* L.) under field and controlled conditions. *J. Plant Biol. Res.,* **1**: 107-112.

Arabi MIE, El-Shehadah E, Jawhar M, 2010. Pathogenic groups identified among isolates of *Rhynchosporium secalis*. *Plant Pathol. J.,* **26**: 260-263.

Chowdhury S, Basu A, Kundu S, 2017. Biotrophy-necrotrophy switch in pathogen evokes differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Sci. Rep.,* **7**: 17251.

Derveaux S, Vandesompele J, Hellemans J, 2010. How to do successful gene expression analysis using real-time PCR. *Methods,* **50**: 227-230.

Doehlemann G, WahlR, Vranes M, De Vries RP, Kämper J, Kahmann R, 2008.Establishment of compatibility in the *Ustilago maydis*/maize pathosystem. *J. Plant Physiol.,* **165***:* 29-40.

El Oirdi M, Bouarab K, 2007. Plant signalling components EDS1 and SGT1 enhance disease caused by the necrotrophic pathogen *Botrytis cinerea*. *New Phytol.,* **175**: 131-139.

Gangwar OP, Bhardwaj SC, Singh GP, Prasad P, Kumar S, 2018. Barley disease and their management: An Indian perspective. *Wheat and Barley Research.***10**: 138-150.

Glazebrook J, 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.,* **43**: 205-227.

Golshani F, Fakheri B A, Behshad E, Vashvaei RM, 2015. PRs proteins and their mechanism in plants. *Biol. Forum. Int., J.,* **7**: 477-495.

Goswami RS, XuJR, Trail F, Hilburn K, Kistler HC, 2006. Genomic analysis of host–pathogen interaction between *Fusarium graminearum* and wheat during early stages of disease development. *Microbiology,* **152**: 1877-1890.

Huang J, Gu M, Lai Z, Fan B, ShiK, Zhou Y H, Yu J Q, Chen Z, 2010. Functional analysis of the *Arabidopsis PAL* gene family in plant growth, development, and response to environmental stress. *Plant Physiol.,* **153**: 1526-1538.

Jawhar M, Shoiab A, Arabi MIE, Al-Daoude A, 2017a. Changes in transcript and protein expression levels in the barley - *Cochliobolus sativus* interaction. *Cereal Res. Comm.,* **45**: 1-10.

Jawhar M, Al-Shehadah E, Shoaib A, Orfi M, Al-Daoude A, 2017b. Changes in salicylic acid and gene expression levels during barley-*Blumeria graminis* interaction. *J. Plant Pathol.,* **99**: 651-656.

Jones P, Ayres PG, 1974. *Rhyncosporium* leaf blotch of barley studied during the subcuticular phase by electron microscopy. *Physiol, Plant Pathol.,* **4**: 229-233.

Kabbage M, Yarden O, Dickman MB, 2015. Pathogenic attributes of *Sclerotinia sclerotiorum*: switching from abiotrophic to necrotrophic lifestyle. *Plant Sci.,* **233**: 53-60.

Kervinen BT, Peltonen S, Teeri TH, Karjalainen R, 1998. Differential expression of phenylalanine ammonia-lyase genes in barley induced by fungal infection or elicitors. *New Phytol.,* **139**: 293-300.

Kumar J, Schafer P, Huckelhoven R, Langen G, Baltruschat H, Stein E, Nagarajan S, Kogel HK, 2002. *Bipolaris sorokiniana*, a cereal pathogen of global concern: cytological and molecular approaches towards better control. *Mol. Plant Pathol.,* **3**: 185-195.

Kumar D, Kirti PB, 2015.Transcriptomic and proteomic analyses of resistant host responses in *Arachis diogoi* challenged with late leaf spot pathogen, *Phaeoisariopsis personata*. *PLoS One,* **10**: e0117559.

Li W, Liu Y, Wang J, He M, Zhou X, Yang C, Chen W, 2015. The durably resistant rice cultivar Digu activates defense gene expression before the full maturation of *Magnaporthe oryzae* appressorium. *Mol. Plant Pathol.,* **17**: 354-368.

Liu T, Hou J, Wang Y, Jin Y, Borth W, Zhao F, Liu F, Hu J, Zuo Y, 2016.Genome-wide identification, classification and expression analysis in fungal–plant interactions of cutinase gene family and functional analysis of a putative *ClCUT7* in *Curvularia lunata*. *Mol. Genet. Genom.,* **291**: 1105-1115.

Livak KJ, Schmittgen TD, 2001.Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) method. *Methods,* **25***:* 402-408.

Nolan T, Hands RE, Bustin SA, 2006.Quantification of mRNA using real-time RT-PCR. *Nat. Prot.,* **1**: 1559-1582.

Shen Q H, Zhou F, Bieri S, Haizel T, Shirasu K, 2003. Recognition specificity and RAR1/SGT1 dependency in barley *Mla* disease resistance alleles to the powdery mildew fungus. *Plant Cell.,* **15**: 732-744.

Shen Y, Liu N, Li C, Wang X, Xu X, Chen W, Xing G, Zheng W, 2017. The early response during the interaction of fungal phytopathogen and host plant. *Open Biol.,* **7**: 170057.

Stephens AE, Gardiner DM, White RG, Munn AL, Manners JM, 2008. Phases of infection and gene expression of *Fusarium graminearum* during crown rot disease of wheat. *Mol Plant Microbe Interact*., **21**: 1571-1581.

Spanu PD, Panstruga R, 2017. Editorial: Biotrophic plant-microbe interactions. *Front. Plant Sci.,* **8**: 192.

Xing L, Qian C, Cao A, Li Y, Jiang Z, Li M, 2013. The Hv-SGT1 gene from *Haynaldia villosa* contribute to resistances towards both biotrophic and hemi-biotrophic pathogens in common wheat (*Triticum aestivum* L.). *PLoS One,* **8**: e72571.

Zhang Z, Henderson C, Perfect E, Carver TW, Thomas BJ, Skamnioti P, Gurr SJ, 2005. Of genes and genomes, needles and haystacks: *Blumeria graminis* and functionality. *Mol.. Plant Pathol.,* **6**: 561-575.

Zhao X, Li C, Yan C, 2019. Transcriptome and proteome analyses of resistant preharvest peanut seed-coat in response to *Aspergillus flavus* infection. *Electron. J. Biotechnol.,* **39**: 82-90.

Zadoks JC, Chang TT, Konzak CF, 1974. A decimal code for the growth stages of cereals. *Weed Res.,* **14**: 415-421.